

Improved Quantification of 8-*epi*-Prostaglandin F_{2α} and F₂-isoprostanes by Gas Chromatography/Triple-stage Quadrupole Mass Spectrometry: Partial Cyclooxygenase-dependent Formation of 8-*epi*-Prostaglandin F_{2α} in Humans

Horst Schweer,* Bernhard Watzler, Hannsjörg W. Seyberth and Rolf M. Nüsing
Children's Hospital, Philipps University Marburg, Deutschhausstrasse 12, D-35033 Marburg, Germany

F₂-isoprostanes are considered to be novel markers of lipid peroxidation. To study the *in vivo* formation of F₂-isoprostanes, an improved method was developed for isotope dilution assays involving gas chromatography/triple-stage quadrupole mass spectrometry (GC/MS/MS) including thin-layer chromatography (TLC) (sum of all F₂-isoprostanes) and high-performance liquid chromatographic (HPLC) purification (prostaglandin F_{2α} (PGF_{2α}) and 8-*epi*-PGF_{2α}). Following the addition of isotopically labeled prostaglandins to urine, the sample was acidified and applied to a C₁₈ cartridge. After elution, prostaglandins were derivatized to pentafluorobenzyl esters and subjected to TLC. A broad zone was scratched off, isoprostanes were eluted and after formation of their trimethylsilyl ether derivatives the sum of F₂-isoprostanes was determined by GC/MS/MS. For the determination of PGF_{2α} and 8-*epi*-PGF_{2α} prior to trimethylsilylation an additional HPLC step was performed and the fractions containing PGF_{2α} and 8-*epi*-PGF_{2α} were analyzed by GC/MS/MS. Using this technique, 8-*epi*-PGF_{2α} concentrations in urine samples as low as 5 pg ml⁻¹ could be determined with high accuracy. The excretion rates of isoprostanes were studied in comparison with the classical prostaglandins in three different groups: healthy adults, healthy children and children with hyper-PGE syndrome (HPS), a pathological situation associated with a stimulated PGE₂ synthesis. F₂-isoprostanes represented the main arachidonic acid metabolites in these groups and 8-*epi*-PGF_{2α} excretion was comparable in its amount to the classical prostanoids. To delineate the cyclooxygenase-catalyzed contribution, the influence of indomethacin, an inhibitor of cyclooxygenases, on F₂-isoprostane formation in healthy adults and in HPS children was analyzed. Significantly decreased excretion rates were observed 2 days after indomethacin administration for all prostanoids, including F₂-isoprostanes and 8-*epi*-PGF_{2α}. However, the suppression of F₂-isoprostanes and 8-*epi*-PGF_{2α} excretion rates was less pronounced in comparison with the classical prostanoids. An improved and reliable method for the determination of F₂-isoprostanes and especially 8-*epi*-PGF_{2α} has been developed. The data obtained on human urine samples indicates a contribution of the cyclooxygenase pathway to the formation of isoprostanes. © 1997 John Wiley & Sons, Ltd.

J. Mass Spectrom. 32, 1362–1370 (1997)

No. of Figures: 6 No. of Tables: 2 No. of Refs: 34

KEYWORDS: prostanoids; 8-*epi*-prostaglandin F_{2α}; isoprostanes; gas chromatography/mass spectrometry; cyclooxygenase; indomethacin

INTRODUCTION

F₂-isoprostanes are metabolites of arachidonic acid which have the same formula as prostaglandin F_{2α} (PGF_{2α}) (Fig. 1). The 64 possible isomers can be explained by four regioisomers, each with eight racemic diastereomers.¹ Among these isomers, 8-*epi*-PGF_{2α} (Fig. 1) has attracted special interest owing to its biological activity as a potent vasoconstrictor of the lung and kidney.^{2–4} Interaction with the thromboxane receptor has been shown,^{4,5} but the existence of a unique recep-

tor is also suggested.^{6,7} Different studies have found evidence for the production of F₂-isoprostanes by free radical-catalyzed peroxidation of arachidonic acid,^{8–11} independent of the cyclooxygenase pathway.^{12,13} Consequently, measurement of urinary levels was considered to be a reliable approach to assess oxidant stress in humans^{14–17} with 8-*epi*-PGF_{2α} as an ideal marker for lipid peroxidation. However, conflicting with this approach, more recently an enzyme-dependent formation via the cyclooxygenases has been reported in human monocytes^{18,19} and also in microsomes isolated from ram seminal vesicles.²⁰ Therefore, the function of 8-*epi*-PGF_{2α} as an index marker for lipid peroxidation has to be reconsidered.

Regarding the discrepancy in the experimental observations, it appears important to use a reliable and highly selective method to determine 8-*epi*-PGF_{2α}

* Correspondence to: H. Schweer, Children's Hospital, Philipps University Marburg, Deutschhausstrasse 12, D-35033 Marburg, Germany.

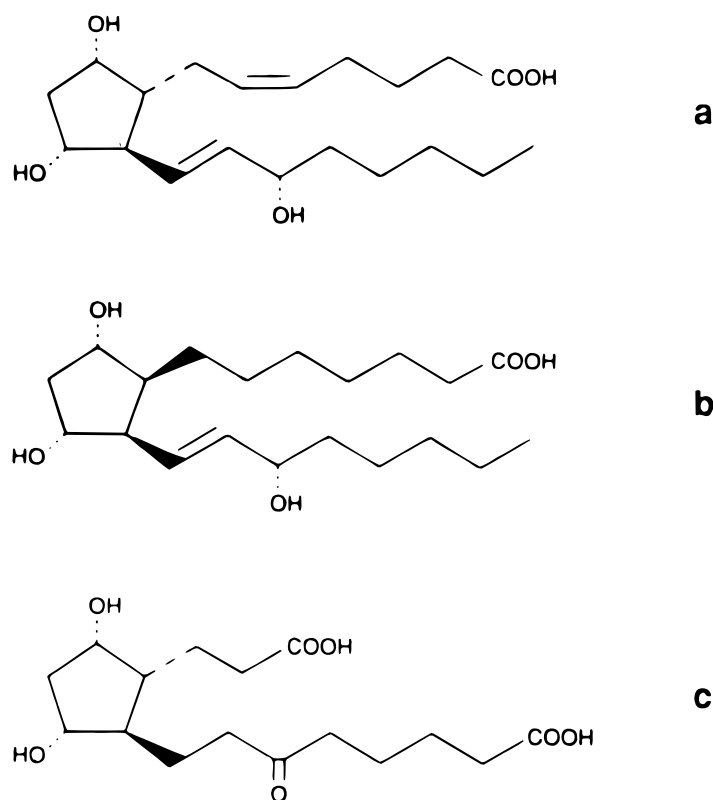


Figure 1. Structures of (a) PGF_{2α}, (b) 8-*epi*-PGF_{2α} and (c) PGF-M, the major urinary metabolite of PGF_{2α}.

concentrations. Different methods using gas chromatographic/mass spectrometric (GC/MS) techniques^{13,21–23} and radioimmunoassay have been described.¹³ However, these methods may give incorrect results owing to co-migrating substances in GC/MS analysis or cross-reactivity of antibodies in immunoassays. To evaluate the *in vivo* formation of F₂-isoprostanes, and especially of 8-*epi*-PGF_{2α}, we established an assay based on high-performance liquid chromatographic (HPLC) purification prior to GC/MS/MS. This unique approach has the highest selectivity of all techniques published so far. By this method the formation of F₂-isoprostanes and 8-*epi*-PGF_{2α} in relation to the synthesis of classical prostanoids was determined in humans before and after application of indomethacin. Our results indicate that at least part of the isoprostanes is formed cyclooxygenase dependent. Using F₂-isoprostanes or 8-*epi*-PGF_{2α} as a lipid peroxidation marker, this needs careful attention.

EXPERIMENTAL

Prostanoids and reagents

[3,3,4,4-²H₄]PGF_{2α}, [3,3,4,4-²H₄]PGE₂, [3,3,4,4-²H₄]-6-keto-PGF_{1α}, [3,3,4,4-²H₄]TxB₂, [18,18,19,19-²H₄]-2,3-dinor-6-keto-PGF_{1α}, [18,18,19,19-²H₄]-2,3-dinor-TxB₂, their non-deuterated analogues and PGE-M were a kind gift from Dr Udo Axen (Upjohn, Kalamazoo, MI, USA). [13,17,17,18,18,19,19-²H₇]PGF-M, [13,17,17,18,18,19,19-²H₇]PGE-M, [18,18,19,19-²H₄]-11-dehydro-TxB₂ and 11-dehydro-TxB₂ were obtained from Dr Claus O. Meese (Dr Margarete-Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart,

Germany). PGF_{2α} and 8-*epi*-PGF_{2α} were obtained from Cayman Chemicals (Ann Arbor, MI, USA). [¹⁸O₂]-8-*epi*-PGF_{2α} was prepared from 8-*epi*-PGF_{2α} and H₂¹⁸O as described elsewhere.²⁴

Ethyl acetate, H₂¹⁸O (96.5%) and chloroform were obtained from Promochem (Wesel, Germany), butyrylcholinesterase from Sigma (Deisenhofen, Germany), *O*-methylhydroxylamine hydrochloride from Serva (Heidelberg, Germany), pentafluorobenzyl bromide from Lancaster (Mühlheim/Main, Germany), water, methanol, ethanol, formic acid and acetonitrile from Merck (Darmstadt, Germany) and hexane and sodium acetate from Riedel-de Haën (Seelze, Germany). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Macherey–Nagel (Düren, Germany) and *N,N*-diisopropylethylamine from Pierce (Oud Beijerland, The Netherlands). Silica gel thin-layer chromatographic (TLC) plates (LK6D, 5 × 20 cm) were obtained from Herolab (Wiesloch, Germany) and helium (99.996%), methane (99.995%), and argon (99.998%) from Messer Griesheim (Herborn, Germany).

Sample preparation

PGE₂, PGE-M, 6-keto-PGF_{1α}, 2,3-dinor-6-keto-PGF_{1α}, TxB₂, 2,3-dinor-TxB₂ and 11-dehydro-TxB₂. Urine and deuterated prostanoids were acidified with formic acid and *O*-methylhydroxylamine hydrochloride in acetate buffer was added to form the methoxime. After acidification with formic acid, the prostanoid derivatives were extracted with ethyl acetate–hexane. After evaporation of the solvent, acetonitrile, pentafluorobenzyl bromide and *N,N*-diisopropylethylamine were added. The mixture was allowed to react at 40 °C for 25 min.

The dry sample was purified by TLC. Three TLC zones containing 6-keto-PGF_{1α} and its major urinary metabolite 2,3-dinor-6-keto-PGF_{1α} (zone 1, R_F 0.03–0.16), PGE₂, TxB₂ and its metabolite 2,3-dinor-TxB₂ (zone 2, R_F 0.17–0.39) and 11-dehydro-TxB₂ and PGE-M (zone 3, R_F 0.40–0.80), the major metabolites of TxB₂ and PGE₂, were scraped off, eluted with the TLC developing solvent and water was added. After centrifugation, the ethyl acetate phase was withdrawn, the solvent was evaporated and the prostanoids were derivatized with BSTFA. A 1 µl aliquot of each solution was injected into the GC/MS/MS system.²⁵

F₂-isoprostanes. Sample preparation was similar to the method described above. As F₂-isoprostanes have no keto function, the methoximation step could be omitted. In the TLC purification step, a broad zone (R_F 0.03–0.30) was scraped off the TLC plate.

PGF_{2α} and 8-*epi*-PGF_{2α}. Urine (1 ml) and labelled prostaglandins ([3,3,4,4-²H₄]PGF_{2α}, 773 pg; [¹⁸O₂]-8-*epi*-PGF_{2α}, 384 pg) were diluted with water (4 ml) and acidified with formic acid (5%, v/v) to pH 3.5. The sample was applied to a C₁₈ cartridge preconditioned with methanol (5 ml) and water (10 ml). The cartridge was washed sequentially with water (10 ml), methanol (4% in water, 10 ml), and hexane (2 × 5 ml). The prostaglandins were eluted with ethyl acetate–hexane (50%, v/v; 10 ml). After evaporation of the solvent, the F₂-isomers were derivatized to the pentafluorobenzyl esters (80 µl of acetone, 6 µl of *N,N*-diisopropylethylamine and 5 µl of pentafluorobenzyl bromide; 10 min at 40 °C) and purified by TLC (ethyl acetate–hexane (9:1, v/v)). A broad zone (R_F 0.02–0.16) was scraped off the TLC plate, eluted with the TLC developing solvent (800 µl) and water (50 µl) was added. After centrifugation, the ethyl acetate phase was withdrawn and the sample was solved in chloroform (80 µl). After addition of tritiated PGF_{2α} pentafluorobenzyl ester (4000 cpm, dissolved in 10 µl, of hexane), the sample was applied to an HPLC column (silica gel; 250 mm × 4 mm i.d.; flow rate 1 ml min⁻¹; eluent, methanol–chloroform (from 0 to 4% (v/v) methanol in 25 min, then from 4 to 5% (v/v) methanol in 20 min and 10% (v/v) methanol for 5 min)). Fractions of 1 ml were collected and the radioactivity of 25 µl samples was determined. Only one sharp peak due to tritiated PGF_{2α} (fraction 36) could be observed. GC/MS/MS of the pentafluorobenzyl ester/trimethylsilyl ether derivatives showed that both PGF_{2α} and 8-*epi*-PGF_{2α} were present in two fractions of the HPLC

eluate (for PGF_{2α}, fractions 35–36; for 8-*epi*-PGF_{2α}, fractions 39–40). The samples were dried under a stream of nitrogen and 25 µl of BSTFA were added to form the trimethylsilyl ether derivatives (1 h, 40 °C). A 1 µl aliquot of this solution was injected into the GC/MS/MS system.

PGF-M

PGF-M (Fig. 1) was determined in an isotope dilution assay by GC/MS/MS as described elsewhere.²⁶

GC/MS/MS

A Finnigan MAT TSQ700 GC/MS/MS system equipped with a Varian Model 3400 gas chromatograph and a CTC A200S autosampler was employed. GC of prostanoid derivatives was carried out on a J&W DB-1 capillary column (20 m × 0.25 mm i.d., 0.25 µm film thickness) (ict, Bad Homburg, Germany) in the splitless mode at an inlet pressure of 100 kPa. The oven temperature program for all prostanoids analyzed was as follows: the initial temperature of 100 °C was held for 2 min, then increased at 30 °C min⁻¹ to 280 °C and at 5 °C min⁻¹ to 310 °C, the final temperature being held for 2 min. The mass spectrometer conditions were as follows: interface temperature, 300 °C; source temperature, 150 °C; methane chemical ionization gas pressure, 50 Pa; electron energy, 70 eV; emission current, 0.4 mA; conversion dynode, 15 kV; and electron multiplier, 1600 V. The collision cell pressure was 0.2 Pa and the collision energy was 12–16 eV. Fragment ions used for quantification were [P – 2(CH₃)₃SiOH][–] (PGE₂), [P – 3(CH₃)₃SiOH][–] (PGF_{2α}, 8-*epi*-PGF_{2α} and F₂-isoprostanes), [P – 3(CH₃)₃SiOH – CH₃OH][–] (6-keto-PGF_{1α}), [P – 3(CH₃)₃SiOH – CH₃OH – CO₂][–] (TxB₂, 2,3-dinor-6-keto-PGF_{1α}, 2,3-dinor-TxB₂), [P – 2(CH₃)₃SiOH – 2CO₂][–] (11-dehydro-TxB₂), [P – C₆F₅CH₂OH][–] (PGF-M) and [P – (CH₃)₃SiOH – C₆F₅CH₂OH][–] (PGE-M), respectively (Table 1).

Human subjects

We investigated urine samples from nine healthy children (seven male, two female; mean age 11.5 years, range 6–15 years), 12 healthy adults (nine female, three male; mean age 24.3 years range 17–38 years) and eight children suffering from hyper-PGE syndrome (HPS) (five male, three female; mean age 10.0 years, range 8–12

Table 1. Parent and fragment ions of endogenous prostanoids and their [²H_n]-analogues ($n = 4-7$) used for quantification

	m/z (endogenous compound)		m/z ([² H _n]-prostanoid)	
	Parent ion	Fragment ion	Parent ion	Fragment ion
11-Dehydro-TxB ₂	511	243	515	247
PGE ₂	524	344	528	348
PGF _{2α} , 8- <i>epi</i> -PGF _{2α} , F ₂ -isoprostanes	569	299	573	303
2,3-Dinor-6-keto-PGF _{1α} , 2,3-dinor-TxB ₂	586	240	590	244
TxB ₂	614	268	618	272
6-Keto-PGF _{1α}	614	312	618	316
PGE-M	637	349	644	356
PGF-M	682	484	689	491

years). HPS is a complex tubular disorder associated with salt wasting, hypercalciuria, isosthenuria, hypokalemic alkalosis, vomiting, diarrhea, fever and failure to thrive.²⁷ Excessive formation of PGE₂, most likely of renal origin, is an important event and is partly responsible for the clinical symptoms. At present, indomethacin application is the most effective therapy. For clinical reasons indomethacin was withdrawn in the subjects with HPS to evaluate the natural history of this disease and the benefit–risk ratio of chronic indomethacin treatment.^{27,28} Prostanoid excretion rates of the adults and the children with HPS were determined with (adults, mean 1.85 mg kg⁻¹ day⁻¹, range 1.6–2.2 mg kg⁻¹ day⁻¹; and children, mean 3.0 mg kg⁻¹ day⁻¹, range 0.9–7.0 mg kg⁻¹ day⁻¹) and without indomethacin medication. In the adults, prostanoid excretion rates were determined before and on the second day of indomethacin administration.

The study protocols were approved by the Ethics Committee of the Philipps University Marburg.

Statistics

The paired *t*-test was used in order to determine whether the changes in prostanoid excretion rates in the eight children and 12 adults with and without indomethacin treatment were significant. Correlations were calculated using Excel 5.0.

RESULTS

For the analysis of the F₂-isoprostane fraction we performed one extraction step and from the TLC plate we scraped off a broad zone (*R_F* 0.03–0.30) prior to GC/MS/MS. This zone contained all F₂-isoprostanes. Analysis of human urine revealed a series of peaks representing F₂-isoprostanes. Typical chromatograms are shown in Fig. 2. Compared with GC/MS,²⁹ our approach utilizing GC/MS/MS reduces the 'biological background' as also reported for determination of classical prostanoids.³⁰ Although the detection of compounds having the same mass as F₂-isoprostanes cannot be excluded completely by this technique, its occurrence is unlikely. There are several peaks in the traces of the endogenous compounds (upper trace, *m/z* 299; retention time (*t_R*) 8:55–9:45 min). The peaks are partly unresolved, indicating that some F₂-isoprostanes may co-chromatograph. For example, the PGF_{2α} peak appears to represent at least three different substances. The treatment of the subject with indomethacin does not affect the peak pattern but the F₂-isoprostane concentration decreased by almost 50% (Fig. 2).

To resolve individual F₂-isoprostanes and to separate 8-*epi*-PGF_{2α} and PGF_{2α} for exact determination, an additional HPLC purification step was included in the sample clean-up. By this procedure a reduced number of peaks in the GC/MS/MS traces allowed the quantification of discrete F₂-isoprostanes. GC/MS/MS analysis

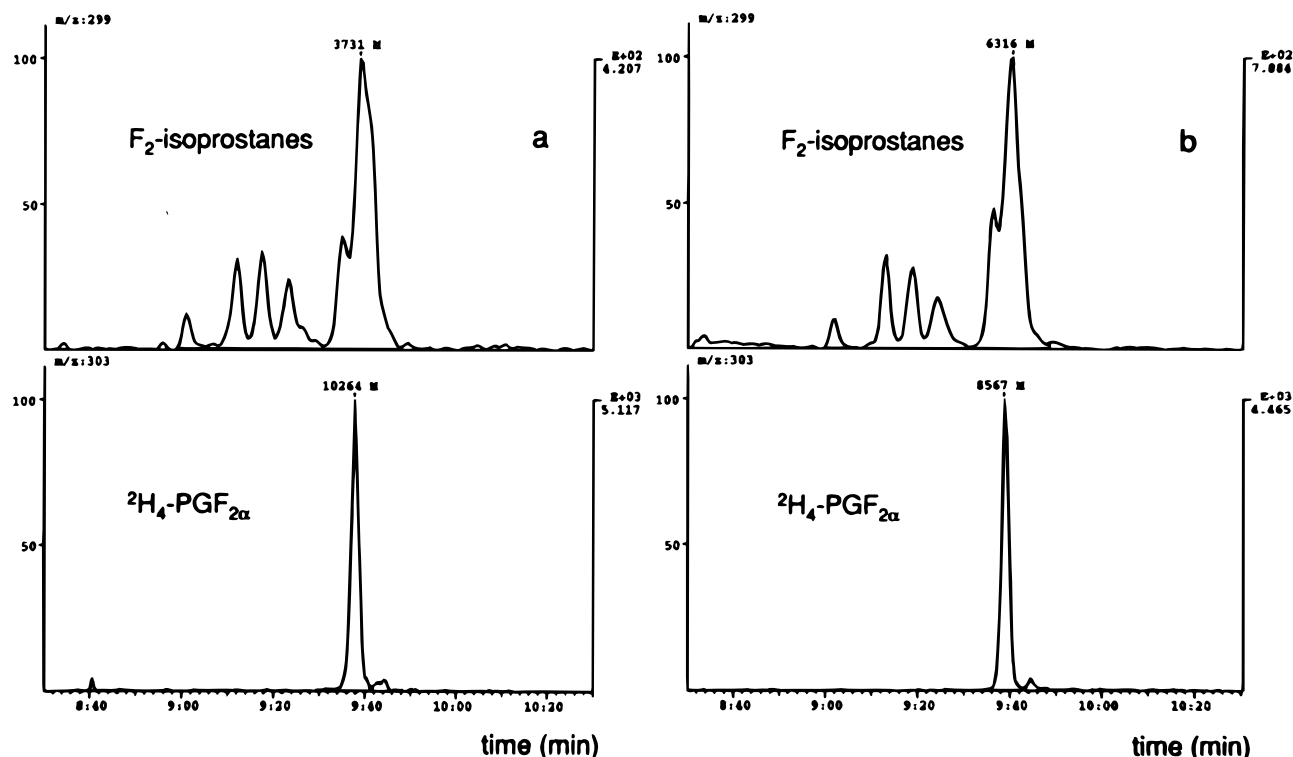


Figure 2. Typical chromatograms of urinary F₂-isoprostanes from subjects with and without indomethacin treatment. The upper traces (*m/z* 299) show multiple unresolved peaks of isoprostanes. Peaks at 9:24 min and 9:39 min co-chromatograph with 8-*epi*-PGF_{2α} and PGF_{2α}, respectively. The lower traces show the peak of the deuterated internal standard ([3,3,4,4-²H₄]PGF_{2α}, *m/z* 303, *t_R* = 9:38 min). The indomethacin treatment does not affect the peak pattern, but F₂-isoprostanes decrease from 6316/8567 area units = 74% (without indomethacin) to 3731/10264 area units = 36% (with indomethacin) of deuterated internal standard ((a) with indomethacin; (b) without indomethacin).

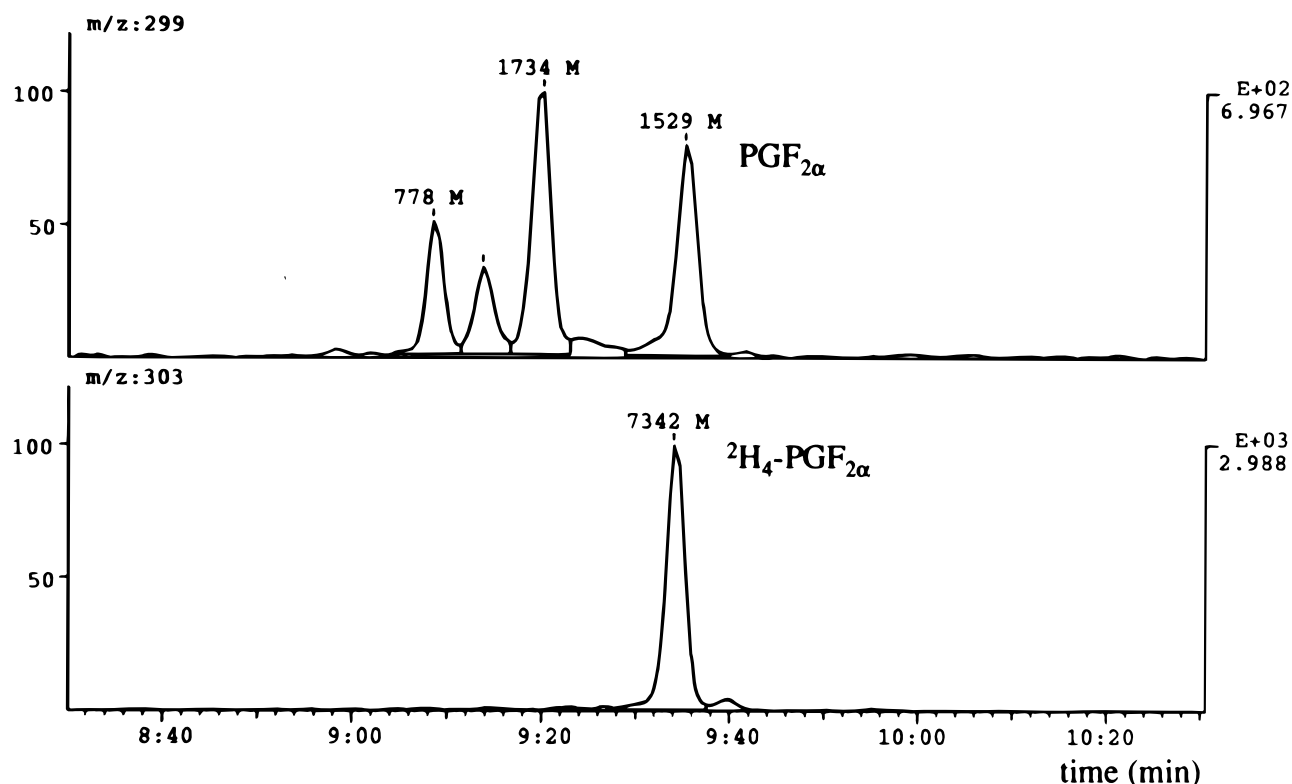


Figure 3. Typical chromatogram of urinary $\text{PGF}_{2\alpha}$. After extraction and HPLC purification, fractions 35 and 36 were combined and quantified by GC/MS/MS. In addition to $\text{PGF}_{2\alpha}$ ($t_R = 9:35$ min), the upper trace (m/z 299) shows three other high isoprostane peaks with $t_R = 9:08$, $9:14$ and $9:21$ min. The peak in the lower trace (m/z 303) belongs to the internal standard $[3,3,4,4\text{-}^2\text{H}_4]\text{PGF}_{2\alpha}$.

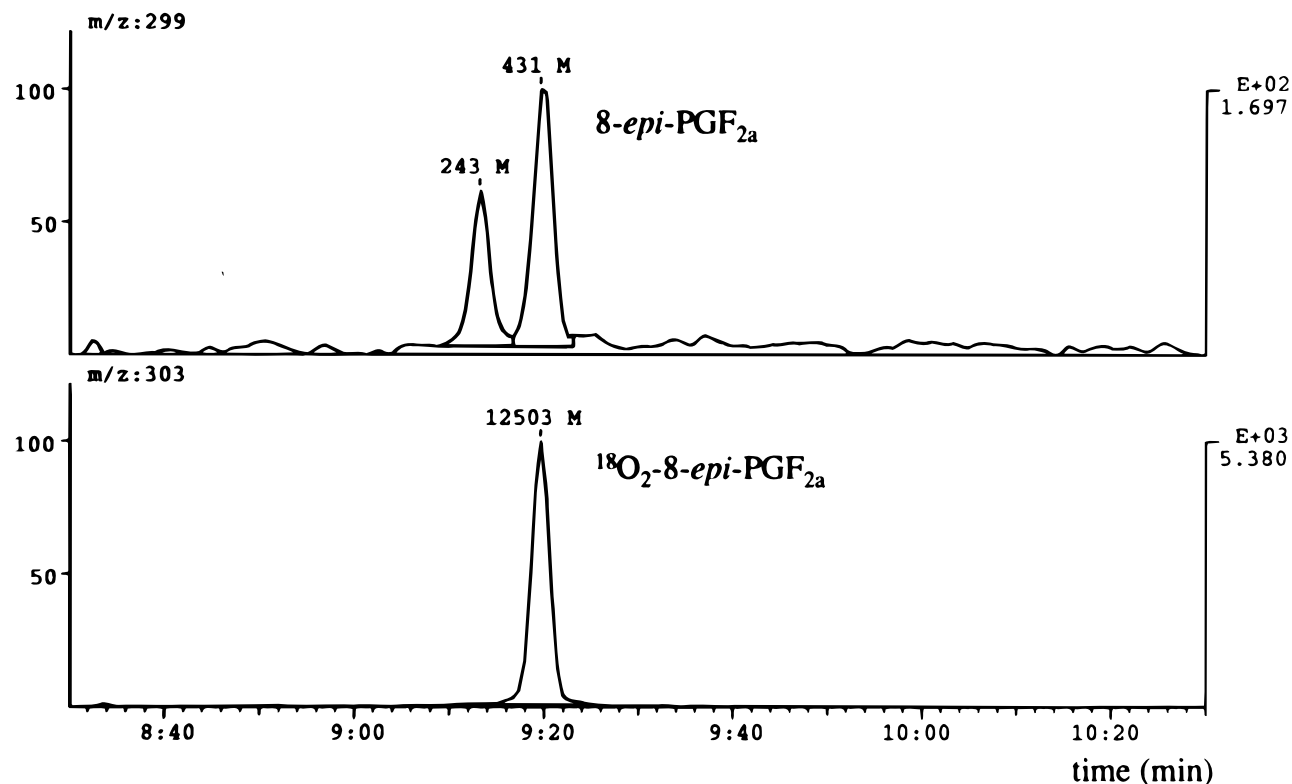


Figure 4. Typical chromatogram of urinary $8\text{-epi-PGF}_{2\alpha}$. After extraction and HPLC purification, fractions 39 and 40 were combined and quantified by GC/MS/MS. In addition to $8\text{-epi-PGF}_{2\alpha}$ ($t_R = 9:20$ min), the upper trace (m/z 299) show only one other F_2 -isoprostane ($t_R = 9:13$ min). The lower trace shows the signal of the internal standard $[^{18}\text{O}_2]\text{-}8\text{-epi-PGF}_{2\alpha}$ ($t_R = 9:20$ min).

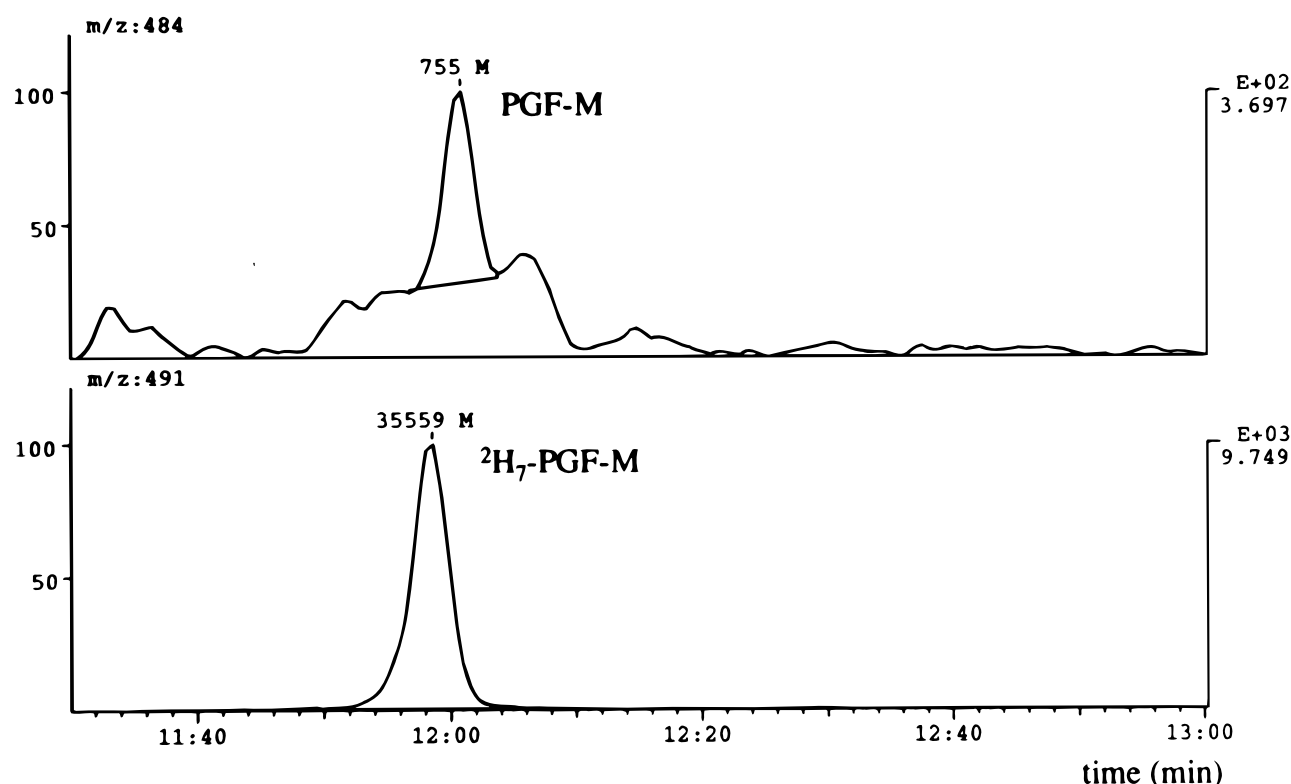


Figure 5. Chromatogram of urinary PGF-M. The upper trace (m/z 484) shows PGF-M (t_R = 12:01 min) as the main peak in the chromatogram, in addition to some other F₂-isoprostane metabolites. The lower trace shows the signal of the internal standard [13,17,17,18,18,19, 19-²H₇]PGF-M (t_R = 11:59 min).

of the HPLC fraction containing radioactively labelled PGF_{2 α} standard (fractions 35–36) revealed the presence of three other major peaks, as yet unidentified, with t_R = 9:08, 9:14 and 9:21 min next to PGF_{2 α} (t_R = 9:35 min; Fig. 3). The peak shape of PGF_{2 α} suggests that no other F₂-isoprostane is co-chromatographing with PGF_{2 α} .

The chromatogram of the HPLC fraction containing 8-*epi*-PGF_{2 α} (fractions 39–40) is shown in Fig. 4. Apart from 8-*epi*-PGF_{2 α} (t_R 9:20 min), another unidentified F₂-isoprostane with t_R = 9:13 min can be observed. Notably, 8-*epi*-PGF_{2 α} and the compound with t_R = 9:21 in Fig. 3 co-chromatograph if a less effective sample clean-up is used. Since the peak in Fig. 3 is four

Table 2. Prostanoid excretion rates (ng h⁻¹ per 1.73 m²) in eight children with HPS (A), 12 healthy adults (B) and nine healthy children (C) and significant differences between excretion rates with and without indomethacin treatment

Subjects	Parameter	PGE ₂	PGE-M	6-Keto-PGF _{1α}	2,3-Dinor-6-keto-PGF _{1α}	TxB ₂	2,3-Dinor-TxB ₂	11-Dehydro-TxB ₂	PGF _{2α}	8- <i>epi</i> -PGF _{2α}	F-Isoprostanes	PGF-M
With indomethacin												
A	Mean	10.2	188	2.7	1.1	0.4	1.8	13.8	24.3	12.8	480	178
	±s.d.	±7.2	±87	±2.6	±2.3	±0.5	±1.3	±9.1	±10.6	±5.7	±262	±106
	Mean	78.7	1184	6.1	4.3	6.0	10.8	108.1	52.8	25.5	801	509
	±s.d.	±64.2	±485	±3.5	±3.5	±6.8	±6.5	±48.3	±19.9	±9.7	±363	±124
Without indomethacin												
B	Mean	8.5	193	4.7	5.1	0.7	3.5	22.7	60.3	24.3	1325	366
	±s.d.	±5.7	±83	±2.8	±4.0	±0.5	±1.8	±5.6	±19.3	±5.3	±341	±117
	Mean	14.4	400	7.9	15.6	2.5	11.3	111.6	83.7	30.9	1605	475
	±s.d.	±7.9	±212	±2.9	±6.5	±2.1	±5.0	±38.1	±24.0	±8.9	±550	±136
Without indomethacin												
C	Mean	15.7	358	4.6	13.2	1.8	11.8	96.9	56.9	27.9	809	376
	±s.d.	±7.2	±62	±1.5	±3.2	±0.3	±5.5	±49.7	±27.4	±20.7	±233	±78
	Mean	15.7	358	4.6	13.2	1.8	11.8	96.9	56.9	27.9	809	376
	±s.d.	±7.2	±62	±1.5	±3.2	±0.3	±5.5	±49.7	±27.4	±20.7	±233	±78

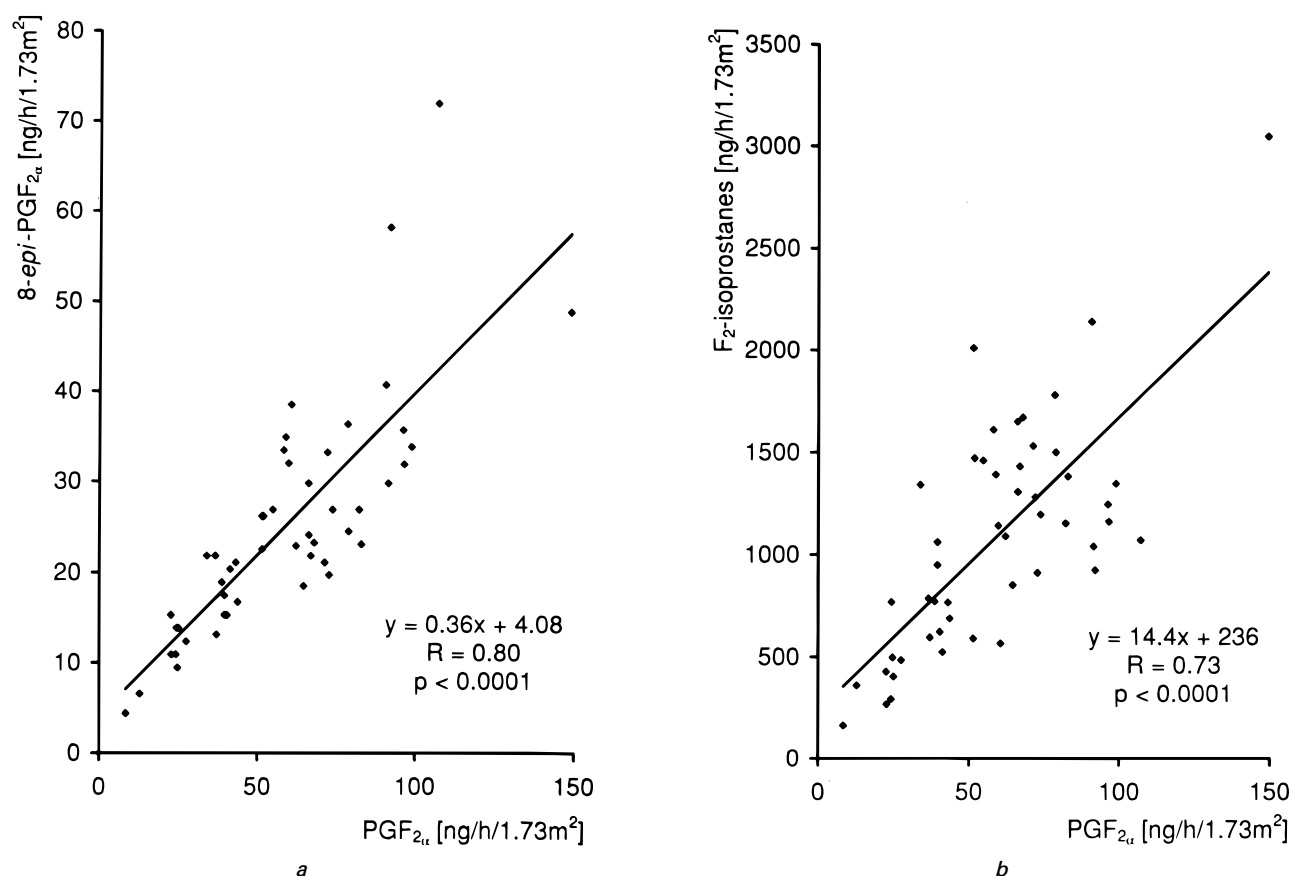


Figure 6. Correlations between (a) PGF_{2α} and 8-*epi*-PGF_{2α} and (b) PGF_{2α} and F₂-isoprostanes. The data are from 12 healthy adults and eight children suffering from HPS (with and without indomethacin medication, respectively) and nine healthy children (without indomethacin medication).

times higher than the corresponding peak in Fig. 2 (1743 compared with 426 area units of 8-*epi*-PGF_{2α}), a method without HPLC purification would overestimate the 8-*epi*-PGF_{2α} concentration.

Metabolization of F₂-isoprostanes results in compounds with a formula identical with that of PGF-M. A chromatogram with endogenous PGF-M ($t_R = 12:01$ min) is shown in Fig. 5. Other F₂-isoprostane metabolites besides PGF-M are present, which is certainly a problem for the quantification of PGF-M.

We used the above-described highly selective method to study the *in vivo* formation of 8-*epi*-PGF_{2α} and F₂-isoprostanes in comparison with the classical prostanoids PGE₂, PGE-M, 6-keto-PGF_{1α}, 2,3-dinor-6-keto-PGF_{1α}, TxB₂, 2,3-dinor-TxB₂, 11-dehydro-TxB₂, PGF_{2α} and PGF-M. Furthermore, to investigate whether a contribution by the cyclooxygenase pathway to 8-*epi*-PGF_{2α} and F₂-isoprostane formation occurs, we analyzed urine samples from healthy adults and HPS patients with application of the COX inhibitor indomethacin. The results are summarized in Table 2.

Our data demonstrate that, first, except for the excretion rates of F₂-isoprostanes which were two times higher in the adult group and for PGE₂ metabolites in children suffering from HPS, all prostanoids including F₂-isoprostanes were formed at similar rates in the groups studied. Second, in all groups we observed a significant reduction in the excretion rates of prostanoids and F₂-isoprostanes on application of indomethacin. Third, relevant differences were observed in the

inhibition of various prostanoids within the groups. The highest sensitivity towards COX inhibition was observed for the TxA₂ products (83–93% in children with HPS and 69–80% in adults); and the lowest sensitivity for the PGF_{2α} products (PGF_{2α} and PGF-M) and the F₂-isoprostanes (40–65% in children with HPS and 18–28% in adults).

Correlations of the excretion rates of PGF_{2α} with those of 8-*epi*-PGF_{2α} and F₂-isoprostanes demonstrated a positive correlation for PGF_{2α} with 8-*epi*-PGF_{2α} ($r = 0.80$, $p < 0.0001$; Fig. 6(a)) and for PGF_{2α} with F₂-isoprostanes ($r = 0.73$, $p < 0.0001$; Fig. 6(b)).

DISCUSSION

For the determination of F₂-isoprostanes we applied a single TLC purification step comparable to reported methods.²¹ To increase the selectivity we used GC/MS/MS instead of GC/MS. This improves the analysis of F₂-isoprostanes. However, one has to bear in mind that also with this technique falsely high values for F₂-isoprostanes may be obtained, as other compounds with the same m/z value may co-chromatograph.

For the determination of 8-*epi*-PGF_{2α}, a more selective prepurification is necessary. After extraction of the prostaglandins on a C₁₈ solid-phase column, Wang *et al.*¹³ used two TLC steps for purification prior to GC/MS. Instead of a second TLC step, we introduced an HPLC purification step, which provides a more

effective clean-up, and for quantification we employed GC/MS/MS instead of GC/MS. We have demonstrated the superiority of GC/MS/MS over GC/MS with respect to selectivity for other prostaglandins.³⁰ Direct comparison of these two methods is not possible, as in the study of Wang *et al.*¹³ no chromatogram was presented and also the zones scraped off the TLC plates were not reported. However, in our experience, the introduction of HPLC in combination with GC/MS/MS greatly improves the accuracy of 8-*epi*-PGF_{2α} determination. At least one compound apart from 8-*epi*-PGF_{2α} was resolved by this technique, which otherwise would lead to the miscalculation of 8-*epi*-PGF_{2α} concentrations. However, owing to a lack of available standards, this compound remains to be identified; to our knowledge, of the 64 isomers of PGF_{2α}, only PGF_{2α} and 8-*epi*-PGF_{2α} are available.

The F₂-isoprostanes are the major prostanoid metabolites in healthy children, adults and children with HPS in comparison with the classical prostanoids and their metabolites. There was no difference in the prostanoid excretion rates between healthy children and healthy adults. Although children with HPS show an impressive increase in the excretion of PGE₂ and its metabolite PGE-M, other prostanoids, including F₂-isoprostanes and 8-*epi*-PGF_{2α}, appear to be formed in similar amounts to those in healthy volunteers. Compared with children, adults exhibit an elevated excretion rate for F₂-isoprostanes. This finding is in agreement with observations of other investigators: in study groups representing subjects of higher age, higher endogenous levels of radicals are found which might be connected with elevated F₂-isoprostane production.¹³ Although little is known about the metabolism of F₂-isoprostanes and 8-*epi*-PGF_{2α}, the detection of these compounds in urine samples most likely indicates a renal origin. This is supported by the fact that 8-*epi*-PGF_{2α} exerts a reduction in glomerular filtration rate and in renal blood flow.⁴

To investigate the contribution of the cyclooxygenase-catalyzed conversion of arachidonic acid to F₂-isoprostanes and especially 8-*epi*-PGF_{2α}, we measured their excretion rates under indomethacin treatment. As expected, nearly complete suppression was obtained for the thromboxane metabolites, indicating the effectiveness of indomethacin treatment. Interestingly, we observed also a small but significant attenuation of the excretion rates of F₂-isoprostanes and 8-*epi*-PGF_{2α} in both the healthy adult and HPS children groups. The apparent higher suppression in the HPS group may be related to the higher doses of indomethacin used for treatment of these children. The greatest drop was found for TxB₂ and its metabolites

and a less pronounced decrease for prostacyclin and PGE₂ products. Similar variations in the inhibition of prostanoids have been reported by others using aspirin.³¹

The highly positive correlations between PGF_{2α} and 8-*epi*-PGF_{2α} and between PGF_{2α} and F₂-isoprostanes may point to a similar mechanism of formation. Consequently, PGF_{2α} must be considered as an F₂-isoprostane and its formation by free radical mechanisms also cannot be excluded. This is supported by our observation that compared with the urinary levels of 8-*epi*-PGF_{2α} and F₂-isoprostanes, the suppression of the level of PGF_{2α} by indomethacin treatment is less pronounced.

Our data suggest that at least in part endogenously formed F₂-isoprostanes and 8-*epi*-PGF_{2α} are synthesized via the cyclooxygenases. As indomethacin is a strong inhibitor of both isoforms of cyclooxygenase, it remains unclear whether both isoforms contribute equally. For human monocytes a correlation between induction of COX-2 expression by LPS and 8-*epi*-PGF_{2α} formation has been demonstrated.¹⁸ We observed that mesangial cells in culture are able to release 8-*epi*-PGF_{2α} and that induction of COX-2 in these cells by interleukin-1 is also associated with an increase in 8-*epi*-PGF_{2α} formation.³² Moreover, in addition to COX-1, basal expression of COX-2 in human kidney has also been reported,^{33,34} indicating that both cyclooxygenases are present and might contribute to the formation of 8-*epi*-PGF_{2α} and F₂-isoprostanes.

In all study groups 8-*epi*-PGF_{2α} is one of the minor components of the F₂-isoprostanes with about 50% of the abundance of PGF_{2α}. This is in contrast to studies by Morrow *et al.*,²¹ who found 8-*epi*-PGF_{2α} to be one of the major F₂-isoprostanes. This discrepancy might be explained by the presence of compounds co-chromatographing with 8-*epi*-PGF_{2α}. Using HPLC in combination with GC/MS/MS, most likely we eliminated these substances.

In conclusion, we have established an improved method using GC/MS/MS in combination with HPLC prepurification for the determination of 8-*epi*-PGF_{2α}. The results demonstrate that, albeit to different extents, the excretion rates of all quantified prostanoids, including 8-*epi*-PGF_{2α} and F₂-isoprostanes, can be reduced by administration of indomethacin.

Acknowledgements

We are grateful to Mrs Daniela Cegledi and Silke Mahr for skilful technical assistance.

REFERENCES

1. J. D. Morrow, T. M. Harris and L. J. Roberts, II, *Anal. Biochem.* **184**, 1 (1990).
2. M. Banerjee, K. H. Kang, J. D. Morrow, L. J. Roberts, II and H. Newman, *Am. J. Physiol.* **263**, H660 (1992).
3. K. H. Kang, J. D. Morrow, L. J. Roberts, II, H. Newman and M. Banerjee, *J. Appl. Physiol.* **74**, 460 (1993).
4. K. T. Takahashi, T. M. Nammour, M. Fukunaga, J. D. Ebert, J. D. Morrow, L. J. Roberts, II, R. L. Hoover and K. F. Badr, *J. Clin. Invest.* **90**, 136 (1992).
5. J. D. Morrow, T. A. Minton and L. J. Roberts, II, *Prostaglandins* **44**, 155 (1992).
6. M. Fukunaga, N. Makita, L. J. Roberts, II, J. D. Morrow,

- K. Takahashi and K. F. Badr, *Am. J. Physiol.* **264**, C1619 (1993).
7. D. E. Pratico, E. M. Smyth, G. Violi and G. A. FitzGerald, *J. Biol. Chem.* **271**, 14916 (1996).
8. J. D. Morrow, J. A. Awad, T. Kato, K. Takahashi, K. F. Badr and L. J. Roberts, II, *J. Clin. Invest.* **90**, 2502 (1992).
9. J. D. Morrow, T. A. Minton, C. R. Mukundan, M. D. Campbell, W. E. Zackert, V. C. Daniel, K. F. Badr, I. A. Blair and L. J. Roberts, II, *J. Biol. Chem.* **269**, 4317 (1994).
10. S. M. Lynch, J. D. Morrow, L. J. Roberts, II and B. Frei, *J. Clin. Invest.* **93**, 998 (1994).
11. A. W. Longmire, L. L. Swift, L. J. Roberts, II, J. A. Awad, R. F. Burk and J. D. Morrow, *Biochem. Pharmacol.* **47**, 1173 (1994).
12. J. D. Morrow, K. E. Hill, R. F. Burk, T. M. Nammour, K. F. Badr and L. J. Roberts II, *Proc. Natl. Acad. Sci. USA* **87**, 9983 (1990).
13. Z. Wang, G. Ciabattoni, C. Créminon, J. A. Lawson, G. A. FitzGerald, C. Patrono and J. Macclouf, *J. Pharmacol. Exp. Ther.* **275**, 94 (1995).
14. M. Reilly, N. Delanty, J. D. Lawson and G. A. FitzGerald, *Circulation* **94**, 19 (1996).
15. N. Delanty, M. Reilly, D. Pratico, D. J. Fitzgerald, J. A. Lawson and G. A. FitzGerald, *Br. J. Clin. Pharmacol.* **42**, 15 (1996).
16. J. D. Morrow, K. P. Moore, J. A. Awad, M. D. Ravenscraft, G. Marini, K. F. Badr, R. Williams and L. J. Roberts, II, *J. Lipid Mediators* **6**, 417 (1993).
17. J. D. Morrow, B. Frei, A. W. Longmire, J. M. Gaziano, S. M. Lynch, Y. Shyr, W. E. Strauss, J. A. Oates and L. J. Roberts, II, *N. Engl. J. Med.* **332**, 1198 (1995).
18. D. Pratico and G. A. FitzGerald, *J. Biol. Chem.* **271**, 8919 (1996).
19. P. Patrignani, G. Santini, M. Panara, M. G. Sciulli, A. Greco, M. T. Rotondo, M. di Giamberardino, J. Macclouf, G. Ciabattoni and C. Patrono, *Br. J. Pharmacol.* **118**, 1285 (1996).
20. M. Hecker, V. Ullrich, C. Fischer and C. O. Meese, *Eur. J. Biochem.* **169**, 113 (1987).
21. J. D. Morrow, T. A. Minton, K. F. Badr and L. J. Roberts, II, *Biochim. Biophys. Acta* **1210**, 244 (1994).
22. J. Nourooz-Zadeh, N. K. Gopaul, S. Barrow, A. I. Mallet and E. E. Ånggård, *J. Chromatogr.* **667**, 199 (1995).
23. D. Pratico, J. A. Lawson and G. A. FitzGerald, *J. Biol. Chem.* **270**, 9800 (1995).
24. W. D. Lehmann, M. Stephan and G. Fürstenberger, *Anal. Biochem.* **204**, 158 (1992).
25. H. Schweer, B. Watzel and H. W. Seyberth, *J. Chromatogr.* **652**, 221 (1994).
26. H. Schweer, C. O. Meese and H. W. Seyberth, *Anal. Biochem.* **189**, 54 (1990).
27. H. W. Seyberth, W. Rascher, H. Schweer, P. G. Kühl, O. Mehls and K. Schärer, *J. Pediatr.* **107**, 694 (1985).
28. A. Leonhardt, G. Timmermanns, B. Roth and H. W. Seyberth, *J. Pediatr.* **120**, 546 (1992).
29. J. D. Morrow and L. J. Roberts, II, *Methods Enzymol.* **233**, 163 (1994).
30. H. Schweer, H. W. Seyberth and R. Schubert, *Biomed. Environ. Mass Spectrom.* **13**, 611 (1986).
31. H. R. Knapp, C. Healy, J. Lawson and G. A. FitzGerald, *Thromb. Res.* **50**, 377 (1988).
32. T. Klein, F. Reutter, H. Schweer, H. W. Seyberth and R. M. Nüsing, *J. Pharmacol. Exp. Ther.* **282**, 1658 (1997).
33. G. P. O'Neill and A. W. Ford-Hutchinson, *FEBS Lett.* **330**, 156 (1993).
34. M. Kömhoff, H. J. Gröne, T. Klein, H. W. Seyberth and R. M. Nüsing, *Am. J. Physiol.* **272**, F460 (1997).